

RESEARCH PAPER

Down-regulation of IL-8 by high-dose vitamin D is specific to hyperinflammatory macrophages and involves mechanisms beyond up-regulation of *DUSP1*

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Received

3 October 2014

Revised

17 June 2015

Accepted

29 June 2015

BACKGROUND AND PURPOSE

There is current interest in vitamin D as a potential anti-inflammatory treatment for chronic inflammatory lung disease, including cystic fibrosis (CF). Vitamin D transcriptionally up-regulates the anti-inflammatory gene *DUSP1*, which partly controls production of the inflammatory chemokine IL-8. IL-8 is overabundant in CF airways, potentially due to hyperinflammatory responses of CF macrophages. We tested the ability of vitamin D metabolites to down-regulate IL-8 production in CF macrophages.

EXPERIMENTAL APPROACH

CF and healthy monocyte-derived macrophages (MDM) were treated with two vitamin D metabolites, 25-hydroxyvitamin D₃ (25OHD₃) and 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), or paricalcitol, synthetic analogue of 1,25(OH)₂D₃. 25OHD₃ was tested at doses of 25–150 nM, whereas 1,25(OH)₂D₃ and paricalcitol at doses of up to 100 nM. IL-8 was stimulated by bacterial virulence factors. As potential anti-inflammatory mechanism of vitamin D metabolites, we assessed up-regulation of *DUSP1*.

KEY RESULTS

MDM from patients with CF and some healthy donors showed excessive production of stimulated IL-8, highlighting their hyperinflammatory phenotype. Vitamin D metabolites down-regulated stimulated IL-8 only in those hyperinflammatory MDM, and only when used at high doses (>100 nM for 25OHD₃, or >1 nM for 1,25(OH)₂D₃ and paricalcitol). The magnitude of IL-8 down-regulation by vitamin D metabolites or paricalcitol was moderate (~30% vs. >70% by low-dose dexamethasone). Transcriptional up-regulation of *DUSP1* by vitamin D metabolites was seen in all tested MDM, regardless of IL-8 down-regulation.

CONCLUSIONS AND IMPLICATIONS

Vitamin D metabolites and their analogues moderately down-regulate IL-8 in hyperinflammatory macrophages, including those from CF. This down-regulation appears to go through *DUSP1*-independent mechanisms.

Abbreviations

25OHD₃, 25-hydroxyvitamin D₃; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; CF, cystic fibrosis; MDM, monocyte-derived macrophage; qPCR, quantitative PCR

Tables of Links

TARGETS
Nuclear hormone receptors^a
Vitamin D receptor
Enzymes^b
CYP24A1
CYP27B1

LIGANDS
1,25(OH) ₂ D ₃ , 1,25-dihydroxyvitamin D ₃
25OHD ₃ , 25-hydroxyvitamin D ₃
Paricalcitol
Vitamin D

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in <http://www.guidetopharmacology.org>, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (^{a,b}Alexander *et al.*, 2013a,b).

Introduction

Vitamin D is a steroid hormone regulating bone health and is also considered to exert beneficial extra-skeletal effects (Holick, 2007), including anti-inflammatory effects. Vitamin D exists in the form of several metabolites, of which the circulating metabolite 25-hydroxyvitamin D₃ (25OHD₃) and the transcriptionally active metabolite 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) are the most relevant ones for biological effects of this hormone. 25OHD₃ is converted by the mitochondrial enzyme CYP27B1 in kidney and other tissues to 1,25(OH)₂D₃ (Figure 1). The latter exerts its biological effects mostly through binding to vitamin D receptor (Figure 1) and up-regulating transcription of vitamin D responsive genes, such as *DUSP1* or *hCAP18* (Figure 1). The effects of 1,25(OH)₂D₃ are limited by its catabolism by the mitochondrial enzyme CYP24A1 (Figure 1).

DUSP1 encodes a homonymous anti-inflammatory phosphatase (Abraham and Clark, 2006; Wang and Liu, 2007). Experimental evidence indicates that anti-inflammatory effects of vitamin D may involve *DUSP1* (Zhang *et al.*, 2012). *DUSP1* partly controls production of the pro-inflammatory chemokine IL-8 (Dauletbaev *et al.*, 2011) and mediates, to some extent, IL-8 down-regulation by corticosteroids (Dauletbaev *et al.*, 2011; Shah *et al.*, 2014). Therefore, it is plausible to assume that vitamin D metabolites could also down-regulate IL-8.

Such down-regulation of IL-8 would benefit many chronic inflammatory lung diseases, including cystic fibrosis (CF). CF is a genetic multi-organ disease with lifelong hyperinflammation in the airways, and IL-8 is overabundant in airway secretions of these patients (Balough *et al.*, 1995; Bonfield *et al.*, 1995; Khan *et al.*, 1995; Rosenfeld *et al.*, 2001;

McGarvey *et al.*, 2002; Kieninger *et al.*, 2013). Although supplementation with vitamin D is routinely recommended for all patients with CF (Tangpricha *et al.*, 2012), this is primarily on order to control CF-associated bone disease (Grey *et al.*, 2008). In contrast, potential ability of vitamin D to modulate inflammation in CF, for instance by down-regulating IL-8 production, has not been systematically tested. It is also unclear whether the recommended circulating levels of 25OHD₃ of 50 nM [Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium, 2011] or 75 nM (Holick *et al.*, 2011) are sufficient to modulate IL-8.

To address these questions, we evaluated the down-regulation of IL-8 by vitamin D metabolites in primary cultures of macrophages from patients with CF and healthy subjects. Macrophages are innate immune cells resident to the airways under physiological conditions and recruited from blood monocytes during inflammation. Macrophages play a pivotal role in bacterial clearance (Lovewell *et al.*, 2014) but may also contribute to airway inflammation (Khan *et al.*, 1995). CF macrophages are considered by many researchers (Kopp *et al.*, 2012; Simonin-Le Jeune *et al.*, 2013; Zhang *et al.*, 2013) as intrinsically hyperinflammatory.

The present study addressed the following three objectives. First, we tested whether macrophages from patients with CF adequately express the genes *CYP27B1*, *VDR* and *CYP24A1* that would be required for down-regulation of IL-8 by vitamin D metabolites. Next, we evaluated how efficiently two vitamin D metabolites, 25OHD₃ and 1,25(OH)₂D₃, would down-regulate IL-8 production in CF macrophages stimulated with bacterial virulence factors. Finally, we examined whether down-regulation of IL-8 by vitamin D metabolites would be associated with up-regulation of *DUSP1*.

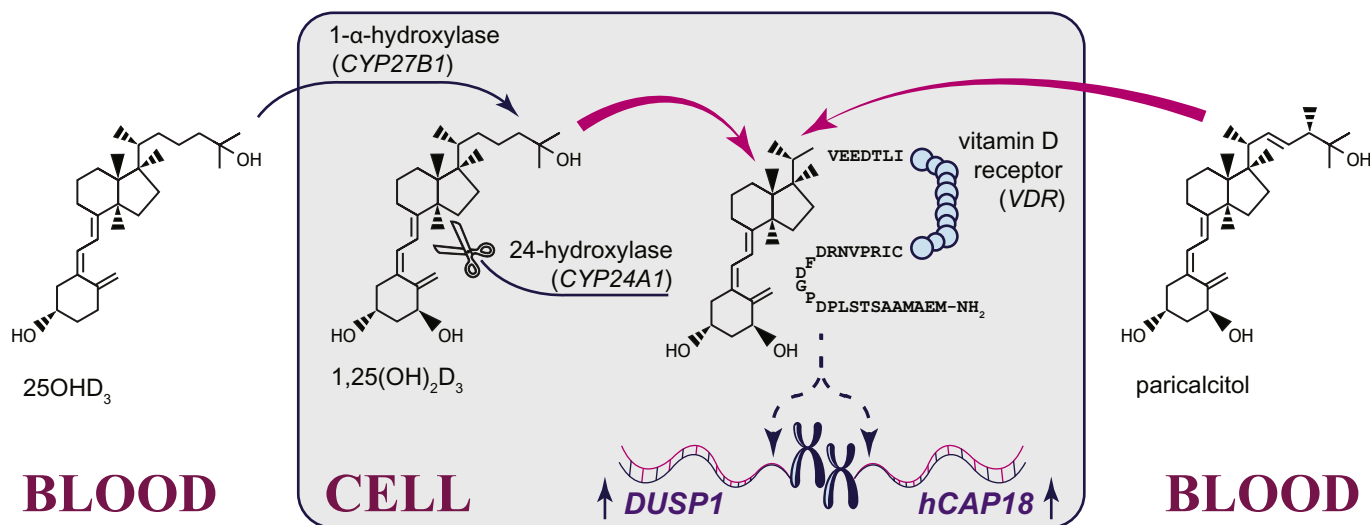


Figure 1

Vitamin D metabolism and signalling. The circulating metabolite of vitamin D (25OHD₃) is converted to the transcriptionally active metabolite (1,25(OH)₂D₃) by the mitochondrial enzyme 1-α-hydroxylase (encoded by the gene *CYP27B1*). 1,25(OH)₂D₃ associates with the vitamin D receptor (gene: *VDR*) and cofactors (not shown), leading to transcriptional up-regulation of vitamin D responsive genes, such as anti-inflammatory *DUSP1* and antibacterial *hCAP18*. The effects of 1,25(OH)₂D₃ are terminated by the mitochondrial enzyme 24-hydroxylase (encoded by the gene *CYP24A1*). Clinically, intracellular levels of 1,25(OH)₂D₃ can be augmented through supplementation with precursors of 25OHD₃ or 25OHD₃ directly. In contrast, direct supplementation with 1,25(OH)₂D₃ is not practical due to hypercalcaemia and hyperphosphatemia caused by high doses of this metabolite. However, low calcemic synthetic analogues of 1,25(OH)₂D₃ (e.g. paricalcitol) can be used to activate the vitamin D receptor and up-regulate transcription of vitamin D responsive genes.

Methods

Isolation of monocytes and culture of monocyte-derived macrophages (MDM)

The use of venous blood from patients with CF or healthy donors to obtain monocytes or MDM was approved by the Human Ethics Board of McGill University Health Centre, and all donors gave written informed consent.

We isolated monocytes as peripheral mononuclear cells from heparin-treated blood using the Ficoll-Paque (GE Healthcare Life Sciences, Mississauga, Canada) protocol. In some experiments, monocytes were further purified using the negative selection Dynabeads Untouched Human Monocytes kit (Life Technologies, Burlington, Canada), according to the manufacturer's instructions. Purified monocytes were used directly in downstream assays, as described below. In the majority of experiments, monocytes were matured to MDM according to the following protocol.

After the Ficoll-Paque isolation, peripheral mononuclear cells were plated onto 96-well plates (Fisher Scientific, Ottawa, Canada) at a concentration of 2.5×10^6 cells·mL⁻¹ and allowed to attach for 3 days in RPMI1640 (Life Technologies) supplemented with 10% normal FBS (Wisent; Saint-Jean-Baptiste, Canada), antibiotics (Life Technologies), and 40 ng·mL⁻¹ of M-CSF (PeproTech; Dollard des Ormeaux, Canada; Figure 2). Then, half the volume of the cell supernatant was removed and replaced with fresh culture medium. After 1 week, culture medium was removed entirely and replaced with RPMI1640 with 10% of either normal or charcoal-depleted FBS (Hyclone, Fisher Scientific), antibiotics,

M-CSF (Figure 2). After 3 days, half volumes of cell supernatants were replaced with fresh culture medium. After another 3 days, cell supernatants were removed entirely, cells were washed once with PBS to remove any remaining non-adherent cells, and supplemented with respective culture medium (Figure 2).

After MDM differentiation for 2 weeks, culture medium was replaced with experimental culture medium and the cells were used in experiments (Figure 2). After a 24 h pretreatment, MDM were stimulated with bacterial virulence factors, as described below, in experimental culture medium (Figure 2). These MDM are equivalent to the M2 macrophage phenotype, prevalent in CF airway secretions (Murphy *et al.*, 2010).

Preparation of sterile filtrates of *Pseudomonas aeruginosa* culture

We used sterile filtrates of *Pseudomonas (P.) aeruginosa* as a stimulus for the majority of the experiments. These filtrates were prepared as described previously (Berube *et al.*, 2010), aliquoted, and stored at -20°C until used for stimulation. In some experiments, we used *P. aeruginosa* flagellin (Invivogen, San Diego, CA, USA), conventional purity and ultrapure *Escherichia (E.) coli* lipopolysaccharide (LPS; Invivogen), and recombinant human IL-1β (BD Biosciences, Mississauga, Canada).

Downstream assays

Plasma samples from healthy individuals and patients with CF were analysed for circulating levels of 25OHD₃ using an

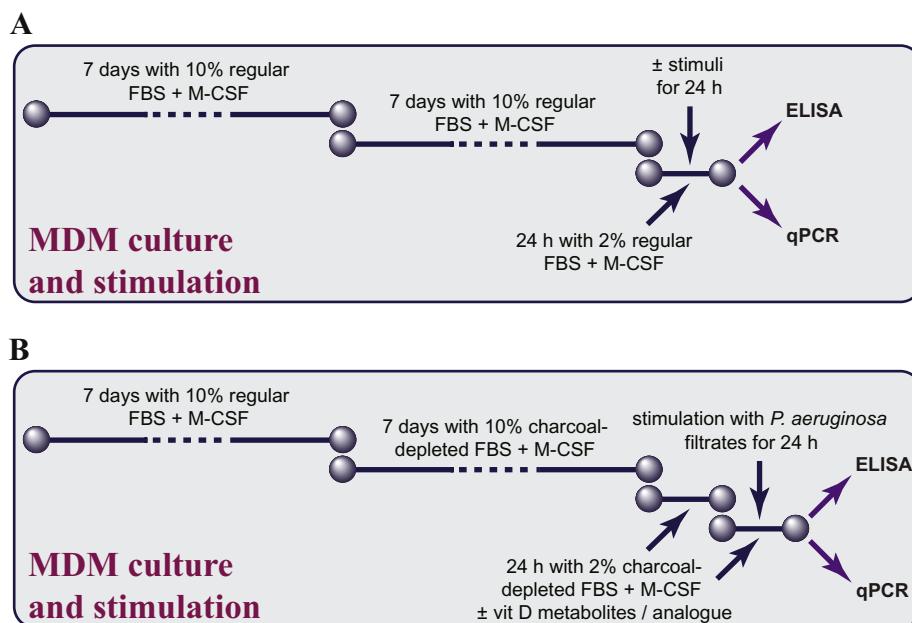


Figure 2

Study design. (A) Study design for experiments testing expression of *VDR*, *CYP27B1* and *CYP24A1*, and experiments testing bacterial virulence factors. MDM were prepared from peripheral blood mononuclear cells and differentiated for 14 days in RPMI1640 supplemented with normal FBS, antibiotics and 40 ng·mL⁻¹ of M-CSF. After maturation, MDM were either collected for qPCR analysis of expression of vitamin D-related genes *VDR*, *CYP27B1* and *CYP24A1*, or stimulated with bacterial virulence factors (stimuli) for 24 h in antibiotic-free RPMI1640 supplemented with 2% normal FBS and M-CSF. Afterwards, cell supernatants were collected for IL-8 ELISA. (B). Study design for experiments testing down-regulation of IL-8 by the two vitamin D metabolites – 25OHD₃ or 1,25(OH)₂D₃ – or the analogue paricalcitol. MDM were prepared from peripheral blood mononuclear cells and differentiated for 7 days in RPMI1640 supplemented with antibiotics, normal FBS and M-CSF. Then, normal FBS was replaced by charcoal-depleted FBS (week 2) to minimize potential confounding effects of vitamin D metabolites present in animal serum. After maturation, MDM were cultured for 24 h in RPMI1640 supplemented with 2% charcoal-depleted FBS, M-CSF, diluent or vitamin D (vit D) metabolites/analogue. Then, MDM received fresh dilutions of the tested compounds and were stimulated for 24 h with diluted (1:100) sterile filtrates of *P. aeruginosa* culture. Afterwards, cell supernatants were collected for IL-8 ELISA, while cells were lysed and subjected to qPCR to quantify expression of *DUSP1* and *hCAP18*.

ELISA assay from Enzo Life Sciences (Farmingdale, NY, USA) according to the manufacturer's instructions.

The IL-8 contents of supernatants from MDM cultures were quantified using an IL-8 ELISA set (BD Biosciences). To account for well-to-well differences in MDM numbers, we used two wells per each experimental condition. Supernatants from replicate wells were analysed separately; average IL-8 values were calculated for each set of replicate wells and used in the final analyses.

For analyses of gene expression, MDM were lysed in lysis buffer RLT (Qiagen, Toronto, Canada). Lysates from duplicate wells were combined to yield sufficient RNA levels, and subjected to RNA isolation (RNeasy Micro kit; Qiagen), reverse transcription (Quantitect RT kit; Qiagen) and qPCR (QuantiFast SYBR Green PCR kit; Qiagen). Most primers for qPCR were from Qiagen. Expression of *CYP27B1* mRNA was quantified using either QuantiFast SYBR Green PCR kit and primers from Qiagen, or TaqMan probe-based primer assay (Life Technologies). Genes of interests were expressed in % of expression of *ACTB*, the gene encoding β -actin. Monocytes isolated by negative selection were lysed, and subjected to RNA isolation, reverse transcription and qPCR, similarly to MDM.

In some experiments, monocytes and MDM were lysed using RIPA buffer: 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 0.1% lauryl sulphate, 0.5% deoxycholic acid, 1% Igepal CA-630, protein inhibitors (Sigma-Aldrich, Mississauga, Canada). These lysates were used in Western blots. Briefly, protein concentrations were quantified and normalized. Samples containing 10 μ g of total protein were first denatured by 1 min incubation at 95°C with denaturing and reducing sample buffer, separated electrophoretically on 10% gels, and transferred onto PVDF membranes (40 V, 90 min). The membranes were blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline (0.1% Tween), incubated overnight at 4°C with a 1:500 diluted rabbit polyclonal antibody against *CYP27B1* (Abcam; Toronto, Canada), washed extensively, probed for 45 min with a 1:5000 diluted goat anti-rabbit secondary antibody (Cell Signaling, Danvers, MA, USA), and washed extensively again. Protein bands were visualized using Western Lightning Plus ECL (Perkin-Elmer, Woodbridge, Canada) and ChemiDoc imaging system (Bio-Rad, Mississauga, Canada). Western blot images were exported as TIFF files to Adobe Photoshop (Creative Suite 5.0; Adobe, San Jose, CA, USA). Only linear

adjustments of contrast and non-obscuring cropping of the bands of interest were applied to those images.

Data analysis

Statistical analyses were conducted using GraphPad Prism (GraphPad Software, La Jolla, CA, USA) and included descriptive statistics, parametric or non-parametric one- or two-way ANOVA comparisons with Tukey or Dunn's *post hoc* tests, or Wilcoxon signed-rank or the Mann–Whitney *U*-test. Absolute values of IL-8 production, IL-8 down-regulation by dexamethasone, and expression of genes of interest in monocytes and MDM were presented as box-and-whisker plots (median, interquartile range and min–max values). Inhibition curves were presented as medians and interquartile ranges of either absolute IL-8 concentrations, or as % of remaining IL-8, versus concentration of the studied compound. The logistic regression analysis of concentration–response to vitamin D metabolites was carried out as previously described (Dauletbaev *et al.*, 2011). The level of statistical confidence was set at $P < 0.05$.

Materials

Most cell culture supplies were from Life Technologies. Synthetic vitamin D metabolites (25OHD₃ and 1,25(OH)₂D₃) were purchased from, respectively, EMD Millipore (Billerica, MA, USA) and Sigma-Aldrich. Their stock solutions (100 µM each) were prepared in 95% ethanol and stored at –20°C. Paricalcitol, a low-calcemic analogue of 1,25(OH)₂D₃, was a kind gift from AbbVie (North Chicago, IL, USA). Its stock solution was prepared at 100 µM in 100% ethanol and stored at –80°C. Dexamethasone was from Sigma-Aldrich; the 1 mM stock solution was prepared in 100% ethanol and stored at

–20°C. Gel electrophoresis supplies (BupH Tris-HEPES-SDS running buffer, BupH Tris-glycine transfer buffer, and 10% Pierce Precise precast protein gels) were from Fisher Scientific, Life Technologies (Magic Mark XP Western Protein Standard), or Bio-Rad. Unless specified, all other supplies were from Sigma-Aldrich.

Results

Expression of vitamin D-related genes in monocytes and MDM

We used cells from 16 healthy individuals (Table 1) and 8 patients with CF (Table 2). Patients with CF were mostly homozygous for the delF508 mutation, which is the most common mutation in Europe and North America. The patients were in clinical remission; all of them received vitamin D supplementation (Table 2).

We first evaluated expression of vitamin D-related genes *CYP27B1*, *VDR* and *CYP24A1* in monocytes and MDM, as both these cell types are targeted by vitamin D metabolites. Both healthy and CF monocytes demonstrated comparable levels of *VDR* expression (respectively Figure 3A, B), while expressions of *CYP27B1* and *CYP24A1* were non-detectable in both monocyte populations (Figure 3A, B). We next assessed MDM for expression of the above genes. MDM expressed *VDR* at the levels comparable to the expression in monocytes (Figure 3C, D vs. Figure 3A, B); there was no difference in expression of *VDR* between healthy and CF MDM (respectively Figure 3C, D). In contrast to monocytes, MDM expressed detectable levels of *CYP27B1* (Figure 3C, D vs. Figure 3A, B), with no detectable difference between healthy

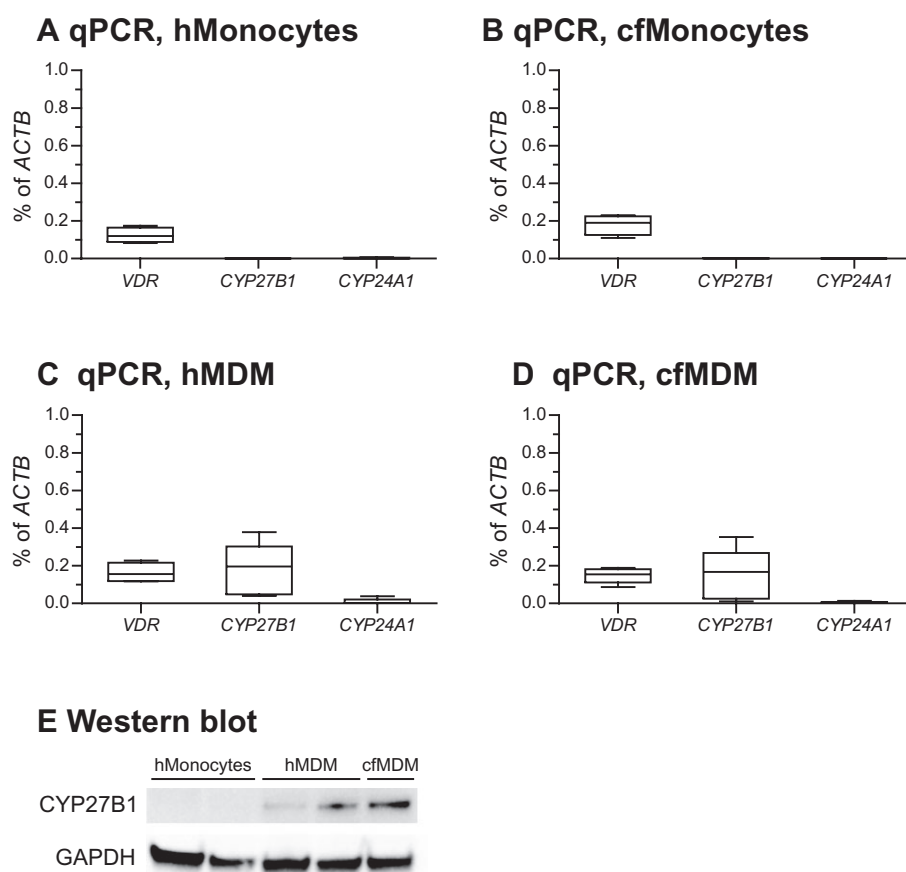
Table 1

Demographic characteristics, enrolment season and plasma levels of 25OHD₃ of healthy individuals

ID	Ethnicity	Enrolment season	Plasma level of 25OHD ₃ (nM)	Vitamin D supplementation
1	Caucasian	Fall	5.3	None
2	Caucasian	Summer	50.8	None
3	Caucasian	Summer	26.8	None
4	Caucasian	Fall	Not enough sample	None
5	Caucasian	Winter	Undetectable	None
6	Caucasian	Fall	32.8	None
7	Caucasian	Summer	44.0	None
8	Caucasian	Summer	94.9	None
9	Caucasian	Summer	32.3	None
10	Caucasian	Fall	33.1	None
11	Caucasian	Fall	130.2	None
12	Caucasian	Fall	50.4	None
13	Caucasian	Fall	40.1	None
14	Caucasian	Winter	59.1	None
15	Caucasian	Summer	Undetectable	None
16	Caucasian	Summer	28.6	None

Table 2Demographic characteristics, enrolment season, and plasma levels of 25OHD₃ of patients with CF

ID (CF genotype)	Age	Ethnicity	Enrolment season	Chronic infection with <i>Pseudomonas aeruginosa</i>	Plasma level of 25OHD ₃ (nM)	Vitamin D supplementation	Pulmonary function (FEV ₁ , % predicted)
1 (delF508/delF508)	26	Caucasian	Fall	No	108	800 IU q.d.	81
2 (delF508/delF508)	29	Caucasian	Fall	Yes	42	1000 IU q.d.	86
3 (delF508/delF508)	25	Caucasian	Fall	Unknown	88	1000 IU q.d.	58
4 (delF508/Y1092X)	28	Caucasian	Fall	Yes	77	400 IU q.d.	29
5 (delF508/G85E)	24	Caucasian	Winter	No	75	1000 IU q.d.	20
6 (delF508/delF508)	31	Caucasian	Winter	Yes	32	1000 IU q.d.	89
7 (delF508/delF508)	27	Caucasian	Spring	Yes	37	1000 IU q.d.	44
8 (delF508/delF508)	26	Caucasian	Spring	Yes	69	1000 IU q.d.	25

**Figure 3**

Expressions of genes related to vitamin D metabolism and signalling. (A and B) Healthy and CF monocytes (hMonocytes and cfMonocytes) were isolated and subjected to qPCR. Expressions of the genes of interest (*VDR*: vitamin D receptor gene; *CYP27B1*: gene encoding 1- α -hydroxylase that converts 25OHD₃ to 1,25(OH)₂D₃; *CYP24A1*: gene encoding 24-hydroxylase that deactivates 1,25(OH)₂D₃) were normalized to expression of *ACTB* (gene encoding β -actin). Data are presented as box-and-whisker plots (medians, interquartile ranges and min–max values). (C and D) MDM were obtained from healthy individuals (hMDM) or patients with CF (cfMDM) and subjected to qPCR. Expressions of the genes of interest were normalized as in (A) and (B). Data are presented as box-and-whisker plots (medians, interquartile ranges and min–max values). (E) A representative Western blot demonstrating intracellular expression of 1- α -hydroxylase (*CYP27B1*) in hMonocytes, hMDM and cfMDM. GAPDH served as a gel-loading control.

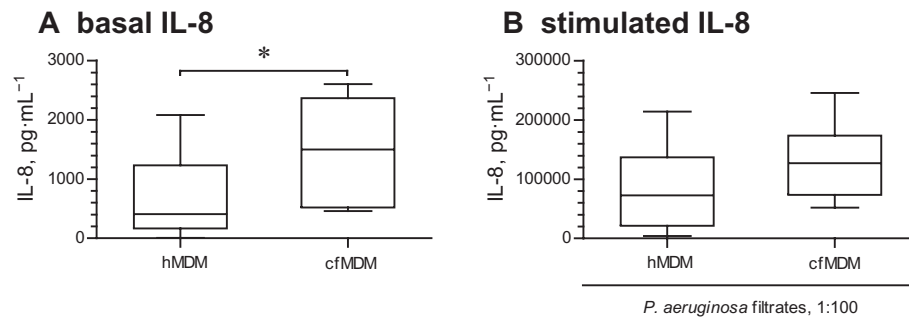


Figure 4

Basal and stimulated IL-8 production. (A) Healthy and CF MDM (hMDM and cfMDM) were tested for basal IL-8 production. IL-8 levels in cell supernatants were quantified by ELISA. * $P < 0.05$. Data are presented as box-and-whisker plots (medians, interquartile ranges and min–max values) of cultures from 16 healthy individuals and 8 patients with CF. (B) The above hMDM and cfMDM were stimulated with diluted (1:100) sterile filtrates of *P. aeruginosa* culture. IL-8 levels in cell supernatants were quantified by ELISA. Data are presented as box-and-whisker plots (medians, interquartile ranges and min–max values).

and CF MDM in expression of this gene. However, MDM expressed *CYP24A1* at low levels, similar to monocytes, (Figure 3C, D). Again, a similar trend was seen in both healthy and CF MDM.

We next confirmed the expression levels of *CYP27B1* using TaqMan probe-based qPCR to warrant higher specificity. TaqMan qPCR yielded results similar to those presented in Figure 3 (data not shown). This indicated that expression of this gene is initiated during monocyte maturation to MDM. This maturation-related phenomenon was also observed by others (Kreutz *et al.*, 1993; Gottfried *et al.*, 2006). We also confirmed the mRNA results at the protein level, comparing expression of corresponding protein in monocytes with that in MDM by Western blot analysis. In agreement with qPCR results, monocytes lacked protein expression, while both healthy and CF MDM expressed it at detectable levels (Figure 3E).

Basal and stimulated IL-8 production in healthy and CF MDM

To test for the hyperinflammatory phenotype of CF MDM, we compared the basal and stimulated IL-8 production between healthy and CF MDM. As a bacterial virulence stimulus, we selected sterile filtrates of *P. aeruginosa*, which stimulated robust secretion of IL-8 in MDM. For the reasons for the use of these filtrates and validating experiments, please refer to Supplementary Results and Supporting Information Fig. S1.

We observed that basal IL-8 production was significantly higher in CF MDM (Figure 4A), compared with healthy MDM, supporting the notion that CF cells may be hyperinflammatory. We next stimulated healthy and CF MDM with sterile *P. aeruginosa* filtrates. Again, the responses of stimulated CF MDM tended to be higher than those of healthy MDM (Figure 4B), indicating that CF MDM exhibit a hyperinflammatory phenotype.

Down-regulation of stimulated IL-8 by 25OHD₃

We next assessed down-regulation of IL-8 by the circulating metabolite 25OHD₃. MDM were supplemented with 25OHD₃

at the physiological (25, 50 and 75 nM) or supraphysiological (100 and 150 nM) concentrations, followed by stimulation with *P. aeruginosa* filtrates. The highest 25OHD₃ concentration of 150 nM was set twofold higher than the maximal recommended concentration (Holick *et al.*, 2011). The inhibitory effects of 25OHD₃ on IL-8 secretion were measured against ethanol as diluent. Ethanol was added to control wells at the same concentration as that in the wells exposed to 150 nM of 25OHD₃.

Analysing the down-regulation of absolute IL-8 by the top concentration of 25OHD₃, we noticed that half of healthy MDM did not down-regulate IL-8, while the other half demonstrated a significant down-regulation of >20% (Figure 5A). Interestingly, the latter MDM, which we dubbed as 'responder' MDM, produced significantly higher levels of stimulated IL-8, compared with 'non-responder' MDM (respective median values of 174635 and 41843 pg·mL⁻¹, $P < 0.01$; Figure 5A). 'Non-responder' MDM mildly, but significantly, up-regulated stimulated IL-8, when treated with 25OHD₃ (Figure 5A). In contrast, 'responder' MDM showed moderate and significant down-regulation of absolute IL-8 levels, especially on 150 nM of 25OHD₃ (Figure 5A). Furthermore, as evident from the analysis of IL-8 levels relative to diluent, treatment with 150 nM of 25OHD₃ mildly up-regulated IL-8 levels in 'non-responder' MDM (Figure 5B), but down-regulated IL-8 in 'responder' MDM by about 30% (Figure 5C).

There was no such heterogeneity of responsiveness to 25OHD₃ in CF MDM samples, as all the CF cells showed a uniform response to 25OHD₃ (Figure 5D). In addition, all tested CF MDM showed high absolute levels of stimulated IL-8 (median value of 126 727 pg·mL⁻¹; Figure 5D), and these were comparable to those of the healthy 'responder' MDM (Figure 5A). In addition, as seen with the healthy 'responder' MDM, CF cells down-regulated their relative production of stimulated IL-8 by nearly 40% when treated with 150 nM of 25OHD₃ (Figure 5E).

Stratification of MDM by their responsiveness to 25OHD₃

The above experiments described above showed that the effects of 25OHD₃ on IL-8 secretion were moderate and

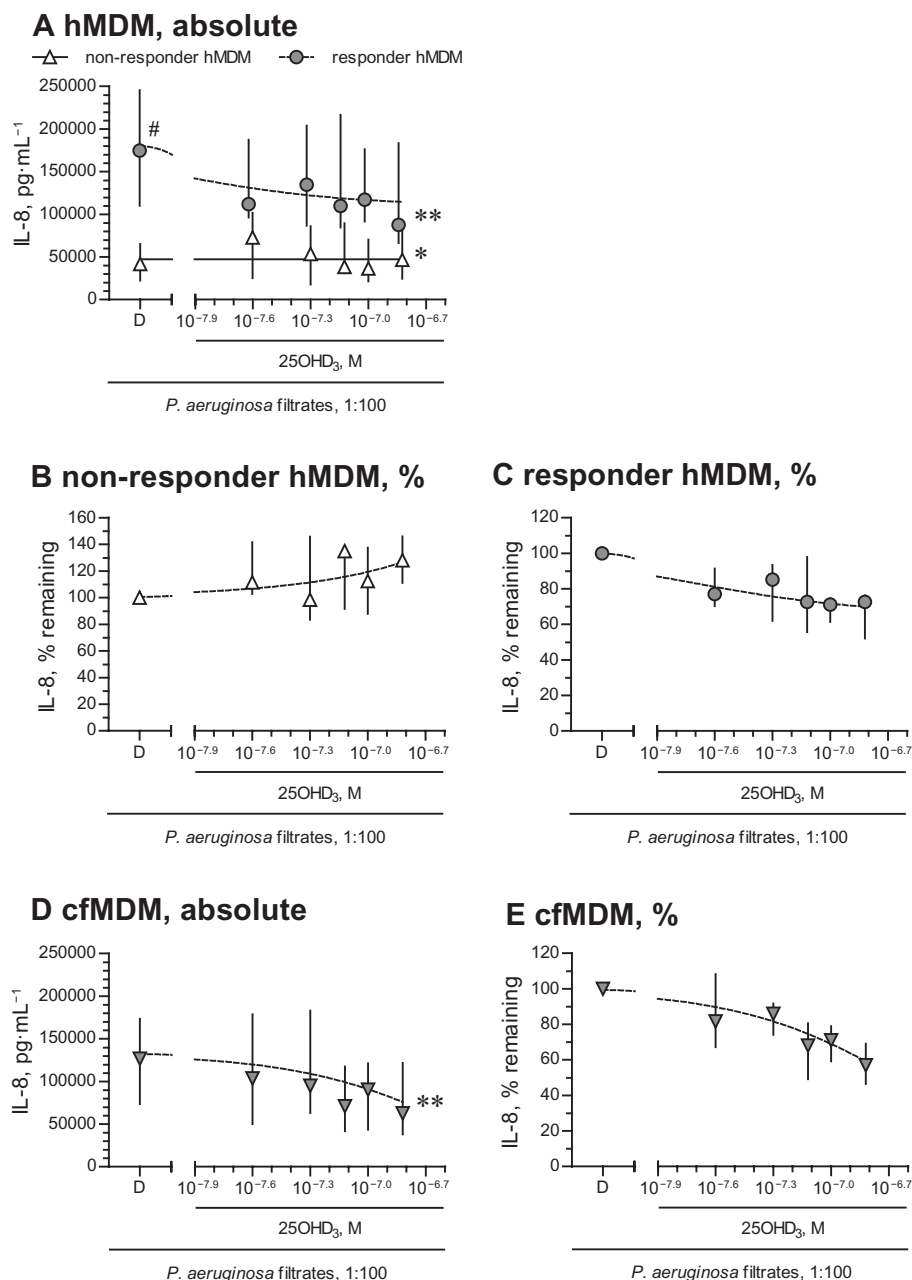


Figure 5

Down-regulation of stimulated IL-8 by 25OHD₃. (A) MDM from 16 healthy individuals (hMDM) were pretreated with diluent (D) or 25OHD₃, and stimulated with diluted (1:100) sterile filtrates of *P. aeruginosa* culture. IL-8 levels in cell supernatants were quantified by ELISA. hMDM from seven healthy individuals that showed a >20% down-regulation of stimulated IL-8 on incubation with the highest concentration of 25OHD₃ (150 nM = 10^{-6.82} M) were defined as 'responder' hMDM, while other (*n* = 8) hMDM with a <20% down-regulation or, in some cases, an up-regulation, were considered as 'non-responder' hMDM. One hMDM culture did not yield sufficient number of wells to test the entire concentration range and was excluded from the analysis. To prevent overlap of error bars, the symbols were slightly set apart along the X-axis on panel (A). Panels (B) and (C) represent down-regulation of stimulated IL-8 relative to diluent in respectively 'non-responder' and 'responder' hMDM. Cultures of MDM obtained from eight patients with CF (cfMDM), were treated in the same way as the hMDM and the corresponding absolute (D) and relative (E) inhibition of stimulated IL-8 release by 25OHD₃ are shown here. Data in panels (A)–(E) are presented as medians and interquartile ranges (symbols and bars); lines are logistic concentration–response curves. #*P* < 0.01, significantly different from non-responder diluent values; **P* < 0.05, ***P* < 0.01; significantly different from corresponding diluent values.

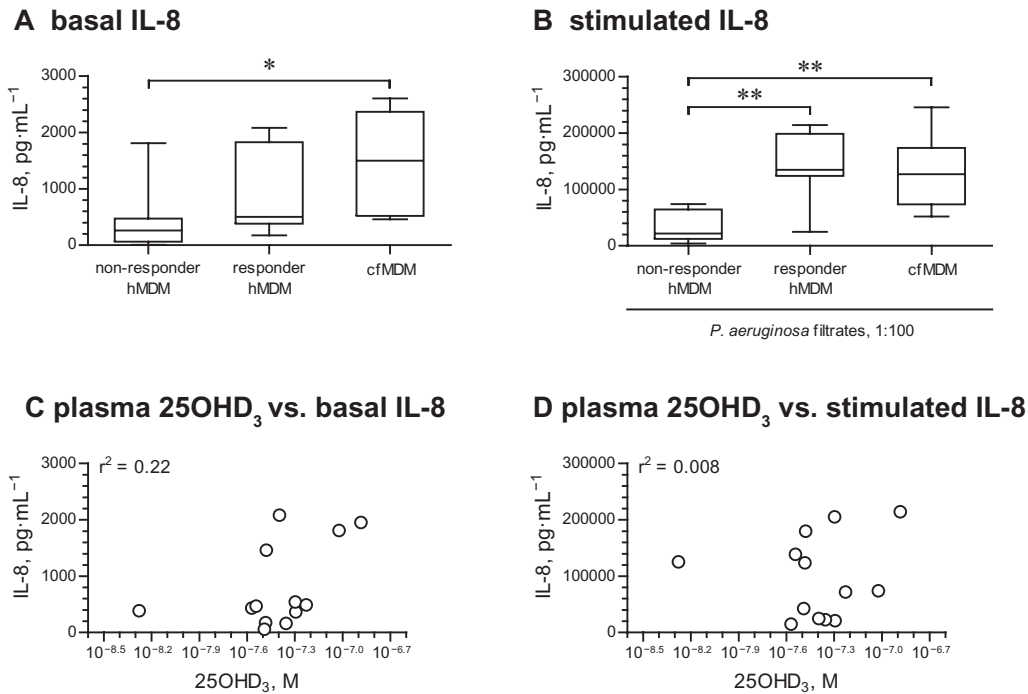


Figure 6

Stratification of healthy MDM on the basis of their responsiveness to IL-8 down-regulating effects of 25OHD₃. (A) Basal IL-8 levels in eight 'non-responder' and seven 'responder' healthy MDM (hMDM), stratified by the inhibition of IL-8 release by 25OHD₃. IL-8 levels in cell supernatants were quantified by ELISA. The responsiveness was defined as a >20% down-regulation of stimulated IL-8 by 150 nM of 25OHD₃ (Figure 5A). * $P < 0.05$. (B) Levels of IL-8 after stimulation with diluted (1:100) sterile filtrates of *P. aeruginosa* culture. The hMDM cultures were stratified based on responsiveness in terms of the down-regulation of IL-8 by 25OHD₃ as defined on panel (A). In both panels (A) and (B), IL-8 production in cfMDM from seven patients with CF is shown as comparison. ** $P < 0.01$. Data on panels (A) and (B) are presented as box-and-whisker plots (medians, interquartile ranges and min-max values). (C) Association between circulating levels of 25OHD₃ in plasma specimens from healthy individuals and basal IL-8 production in their MDM. The levels of 25OHD₃ in plasma specimens and IL-8 in cell supernatants were quantified by respective ELISAs. Two plasma specimens yielded 25OHD₃ levels greatly below the level of detection of the ELISA and were excluded from the analysis. One healthy individual did not produce blood specimen to obtain sufficient quantity of plasma for 25OHD₃ ELISA. Thus, the final analysis included cultures from 13 healthy subjects with plasma levels of 25OHD₃ ranging from 5.3 nM ($10^{-8.23}$ M) to 130 nM ($10^{-6.89}$ M). (D) Association between circulating levels of 25OHD₃ in plasma specimens from healthy individuals and IL-8 production stimulated with diluted (1:100) sterile filtrates of *P. aeruginosa* culture. All MDM cultures and analyses were as in (C).

individualized, in that they were present only in MDM that produced high levels of IL-8 when stimulated with *P. aeruginosa* filtrates (i.e. hyperinflammatory MDM). To further explore the association between the magnitude of stimulated IL-8 production and responsiveness to 25OHD₃, we stratified basal and stimulated output of IL-8 from healthy MDM, based on their responsiveness to 25OHD₃.

According to this stratification, healthy 'responder' MDM tended to exhibit higher basal IL-8 production (Figure 6A) and significantly higher values of stimulated IL-8 (Figure 6B), compared with healthy 'non-responder' MDM (respectively Figure 6A, B). Further supporting an association between the magnitude of stimulated IL-8 and responsiveness to 25OHD₃, healthy 'responder' MDM produced IL-8 at the levels comparable to those of CF MDM (Figure 6B).

We next tested whether there was an association between circulating levels of 25OHD₃ in the blood samples from which the MDM were derived, and IL-8 production in these MDM. We measured the levels of 25OHD₃ in blood plasma of the healthy donors (Table 1), and plotted them against basal (Figure 6C) and stimulated (*P. aeruginosa* filtrates; Figure 6D)

IL-8 production by their MDM. As shown, circulating levels of 25OHD₃ did not predict subsequent IL-8 production of healthy MDM. Measurements in the CF samples showed a similar lack of correlation between endogenous 25OHD₃ levels and output of IL-8 from MDM (data not shown).

Down-regulation of IL-8 by 1,25(OH)₂D₃ or its synthetic analogue paricalcitol

In the preceding set of experiments, stimulated IL-8 production in 'non-responder' MDM was not affected by 25OHD₃. It was possible that this reflected less efficient conversion of 25OHD₃ to transcriptionally active 1,25(OH)₂D₃ in 'non-responder' MDM. To test this, we treated MDM with 1,25(OH)₂D₃, the transcriptionally active metabolite, before stimulation with the filtrates of *P. aeruginosa*. CF MDM were not tested here, as all samples of these cells were 'responders' to 25OHD₃ in the preceding experiments.

As before, we observed two distinct populations of MDM, either responding or not responding to 1,25(OH)₂D₃, in terms of IL-8 release. Similar to previous findings, 'non-responder' MDM exhibited lower absolute production of stimulated IL-8

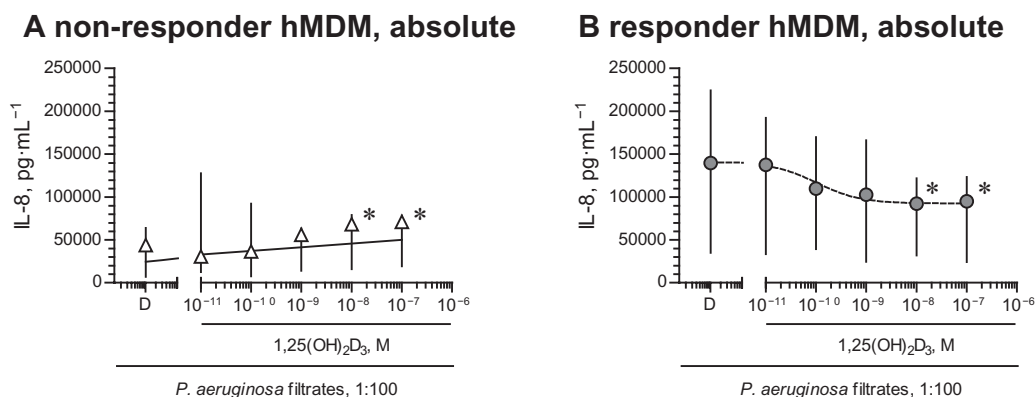


Figure 7

Down-regulation of stimulated IL-8 by 1,25(OH)₂D₃. (A and B) Healthy 'non-responder' ($n = 7$) and 'responder' ($n = 7$) MDM (hMDM) were pretreated with diluent (D) or 1,25(OH)₂D₃ and stimulated with diluted (1:100) sterile filtrates of *P. aeruginosa* culture. IL-8 levels in cell supernatants were quantified by ELISA and normalized to IL-8 levels of cells treated with diluent (D). Definition of 'non-responder' and 'responder' hMDM was as in Figure 5. Data in panels (A) and (B) are presented as medians and interquartile ranges (respectively symbols and bars); lines are logistic concentration–response curves. * $P < 0.05$, significantly different from corresponding diluent values.

(Figures 7A and B). Furthermore, as with 25OHD₃, 'non-responder' MDM showed some up-regulation of IL-8 secretion when treated with 1,25(OH)₂D₃ (10 and 100 nM; Figure 7A). In contrast, 'responder' MDM uniformly showed down-regulation of stimulated IL-8 (Figure 7B), with the greatest effect of about 40% inhibition, at 100 nM 1,25(OH)₂D₃.

Importantly, in 'responder' MDM, release of stimulated IL-8 was inhibited by 1,25(OH)₂D₃ at concentrations equal to or greater than 1 nM (Figure 7B). These concentrations exceed the levels of 1,25(OH)₂D₃ typically observed in blood (Lips, 2007; Biancuzzo *et al.*, 2013) by at least two orders of magnitude. It is not recommended clinically to use 1,25(OH)₂D₃ at such concentrations range because of the potential side effects of hypercalcaemia and hyperphosphatemia. However, these adverse effects are less pronounced in low calcemic analogues of 1,25(OH)₂D₃, such as paricalcitol, and these analogues offer an alternative to 1,25(OH)₂D₃ for supplementation in clinical settings. We next tested the effects of paricalcitol on the stimulated release of IL-8. When tested in three 'responder' MDM cultures, paricalcitol down-regulated IL-8 release similarly to 1,25(OH)₂D₃. Thus, median levels of stimulated IL-8 were decreased from 219 989 pg·mL⁻¹ (diluent treated MDM) to 165 327 pg·mL⁻¹ (10 nM paricalcitol) or to 157 731 pg·mL⁻¹ (100 nM paricalcitol). This was equivalent to about 40% inhibition of the control (diluent only) release (inhibitory curves not shown).

Up-regulation of *hCAP18* as a measure of transcriptional activity of 25OHD₃ and 1,25(OH)₂D₃

The data from the preceding set of experiments demonstrated that the transcriptionally active metabolite 1,25(OH)₂D₃ did not down-regulate IL-8 in 'non-responder' MDM. We therefore wanted to exclude the possibility of abnormal vitamin D signalling in these cells. To this end, we next assessed

up-regulation of expression of the vitamin D responsive gene, *hCAP18*, as a measure of cell responsiveness to vitamin D metabolites.

Up-regulation of *hCAP18* by 25OHD₃ and 1,25(OH)₂D₃ was quantified in the presence of *P. aeruginosa* filtrates to exclude potential negative regulation of cell responsiveness to vitamin D metabolites by bacterial virulence factors. The filtrates alone mildly up-regulated expression of *hCAP18* (<2-fold increase; data not shown). We further observed that in the presence of *P. aeruginosa* filtrates, *hCAP18* was potently and concentration-dependently up-regulated by 1,25(OH)₂D₃ in both 'non-responder' and 'responder' MDM (Figure 8A). The relative up-regulation of *hCAP18* greatly exceeded one order of magnitude in both 'non-responder' and 'responder' MDM, and was at its maximum at 100 nM 1,25(OH)₂D₃ (Figure 8A). The magnitude of relative up-regulation of *hCAP18* tended to be even higher in the 'non-responder' compared with 'responder' MDM (respective median increases of 155- and 78-fold).

We next tested the magnitude of *hCAP18* up-regulation by 25OHD₃. To this end, we incubated CF MDM, all of which were responsive to 25OHD₃ in preceding experiments, with sterile filtrates of *P. aeruginosa* culture and 25OHD₃. As expected, 25OHD₃ also very efficiently up-regulated expression of *hCAP18*, with the most dramatic up-regulation at concentrations of 75 nM and above (Figure 8B). Currently, 75 nM is the highest concentration recommended for optimal bone health (Holick *et al.*, 2011).

Up-regulation of *DUSP1* by 1,25(OH)₂D₃ in 'non-responder' and 'responder' MDM

The previous experiments revealed that 'non-responder' MDM fully retained their responsiveness to transcriptional effects of two vitamin D metabolites, 25OHD₃ and 1,25(OH)₂D₃, as judged by up-regulation of *hCAP18*. *DUSP1* is another vitamin D responsive gene, which partly controls stimulated IL-8 production (Dauletbaev *et al.*, 2011), and

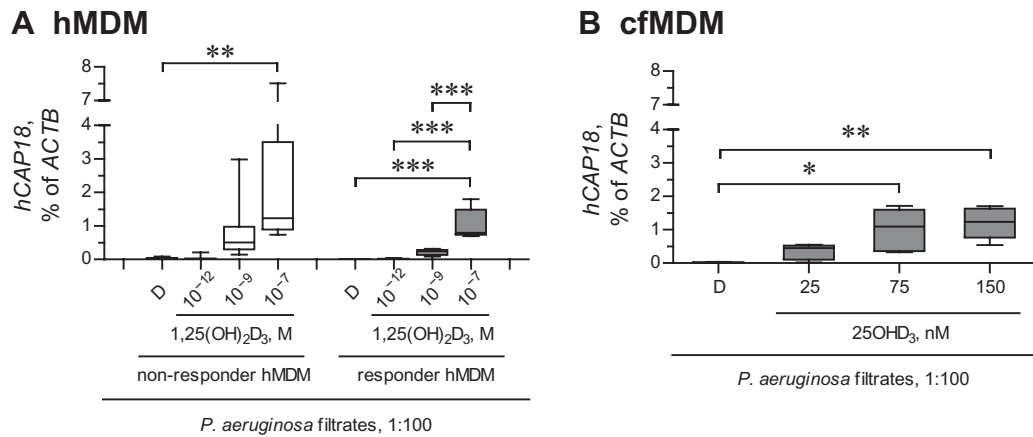


Figure 8

Up-regulation of *hCAP18* expression by two vitamin D metabolites 25OHD₃ and 1,25(OH)₂D₃ in 'non-responder' and 'responder' MDM. (A) Healthy 'non-responder' and 'responder' MDM (hMDM) were pretreated with diluent (D) or 1,25(OH)₂D₃, and stimulated for 24 h with diluted (1:100) sterile filtrates of *P. aeruginosa* culture in the presence of D or 1,25(OH)₂D₃. Definition of 'non-responder' and 'responder' hMDM is given in Figure 6. Expression of the vitamin responsive gene *hCAP18* was quantified by qPCR and normalized to expression of *ACTB*, the gene encoding β-actin. ***P* < 0.01, ****P* < 0.001; significantly different as indicated. (B) cfMDM were pretreated with diluent (D) or 25OHD₃, and stimulated with diluted (1:100) *P. aeruginosa* filtrates. Expressions of *hCAP18* and *ACTB* were quantified as in panel (A). **P* < 0.05, ***P* < 0.01; significantly different as indicated. Data in panels (A) and (B) are presented as box-and-whisker plots (medians, interquartile ranges and min-max values).

up-regulation of *DUSP1* could represent a mechanism for the down-regulation of IL-8 by vitamin D metabolites. Therefore, in the next experiment, we tested basal expression of *DUSP1* and the magnitude of up-regulation of this gene by 1,25(OH)₂D₃, in both 'non-responder' and 'responder' MDM. Basal expression of *DUSP1* was comparable between 'non-responder' and 'responder' MDM (Figure 9). Furthermore, 1,25(OH)₂D₃ up-regulated *DUSP1* with comparable magnitudes in both 'non-responder' and 'responder' MDM (Figure 9).

'Non-responder' MDM down-regulate stimulated IL-8 when treated with low-dose dexamethasone

Finally, we wanted to establish whether the lack of down-regulation of stimulated IL-8 in 'non-responder' MDM was specific to vitamin D metabolites. We thus treated both 'non-responder' and 'responder' MDM with a low concentration (5 nM) of dexamethasone, a potent anti-inflammatory corticosteroid. We found that 'non-responder' MDM, in general, retained their responsiveness to dexamethasone by down-regulating IL-8 production (Supporting Information Fig. S2). Even though the magnitude of IL-8 down-regulation was slightly less pronounced in 'non-responder' MDM (median down-regulation of 72% vs. 83% in 'responder' MDM), dexamethasone down-regulated stimulated IL-8 far more effectively than vitamin D metabolites or their analogues, and this was true for both tested MDM populations (dexamethasone-induced down-regulation: <70% in all tested MDM; vitamin D metabolites/analogues: ~30% in 'responder' MDM and none in 'non-responder' MDM).

Discussion

In this study, we tested whether primary cultures of macrophages from patients with CF, modelled by their MDM,

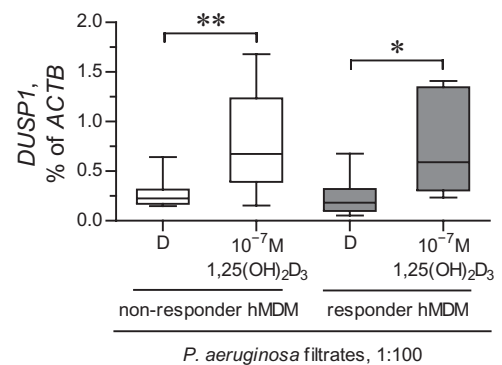


Figure 9

Up-regulation of *DUSP1* expression by vitamin D metabolites 25OHD₃ and 1,25(OH)₂D₃ in 'non-responder' and 'responder' MDM. Healthy 'non-responder' and 'responder' MDM (hMDM) were pretreated with diluent (D) or 1,25(OH)₂D₃, and stimulated for 24 h with diluted (1:100) sterile filtrates of *Pseudomonas aeruginosa* culture in the presence of D or 1,25(OH)₂D₃. Definition of 'non-responder' and 'responder' hMDM was as in Figure 6. Expression of the vitamin responsive gene *DUSP1* was quantified by qPCR and normalized to expression of *ACTB*, the gene encoding β-actin. **P* < 0.05, ***P* < 0.01, ****P* < 0.001; significantly different as indicated. Data are presented as box-and-whisker plots (medians, interquartile ranges and min-max values).

expressed sufficient levels of vitamin D-related genes (*CYP27B1*, *VDR* and *CYP24A1*) to benefit from the possible down-regulation of IL-8 by two vitamin D metabolites, 25OHD₃ and 1,25(OH)₂D₃, and an analogue. It was further assessed to what extent these metabolites down-regulate IL-8 in cells stimulated with bacterial virulence factors. We also tested whether the down-regulation of IL-8 by vitamin D

metabolites involved up-regulation of the vitamin D responsive anti-inflammatory gene *DUSP1*.

Epidemiological studies highlight an association between circulating levels of 25OHD₃ and various disease outcomes in CF (McCauley *et al.*, 2014; Simoneau *et al.*, 2014; Sexauer *et al.*, 2015; Vanstone *et al.*, 2015). Studying this subject more closely, there has been an interventional study (Grossmann *et al.*, 2012) and an *in vitro* study with immortalized airway epithelial cell lines (McNally *et al.*, 2011) that tested modulation of inflammatory cytokines by vitamin D metabolites. There has been no study to date that tested the anti-inflammatory effects of vitamin D metabolites in macrophages, which are important innate immunity cells. Furthermore, to our knowledge, whether CF cells adequately express the genes involved in metabolism, signalling, and catabolism of 25OHD₃ and 1,25(OH)₂D₃ had never been tested.

Here we have presented evidence that CF MDM expressed normal levels of vitamin D-related genes. Furthermore, CF MDM, similar to healthy MDM, expressed the gene *CYP27B1* to convert 25OHD₃ to 1,25(OH)₂D₃. Supporting this, our subsequent experiments demonstrated that CF MDM respond to 25OHD₃ by up-regulating the vitamin D responsive gene, *hCAP18*, and down-regulating the stimulated release of IL-8.

IL-8 down-regulation by vitamin D metabolites and their analogue in our cell culture experiments was achievable only when high concentrations were applied. Furthermore, the MDM that were most responsive to down-regulation of IL-8 by vitamin D metabolites exhibited a hyperinflammatory phenotype, defined by a higher level of IL-8 release when stimulated with bacterial virulence factors. These hyperinflammatory MDM included all the samples from patients with CF and, surprisingly, from some healthy individuals. The hyperinflammatory MDM phenotype was not associated with a diminished expression of *DUSP1* or the magnitude of up-regulation of this gene by 1,25(OH)₂D₃.

As the responsiveness to down-regulation of IL-8 by 25OHD₃ and 1,25(OH)₂D₃ was individualized, it was necessary to identify its determinants. To this end, in the present study we have excluded transcriptional up-regulation of *DUSP1* as one such determinant.

The concentrations of vitamin D metabolites necessary to achieve IL-8 down-regulation *in vitro* were much higher than those currently recommended to maintain proper bone metabolism *in vivo*. Specifically, the maximal recommended blood concentration of 25OHD₃ is either 50 or 75 nM, depending on the guidelines. However, in our study, at least 100 nM of 25OHD₃ was needed to down-regulate stimulated IL-8 in MDM. Consistent with this, the concentrations of 1,25(OH)₂D₃ that down-regulated the stimulated release of IL-8 were also substantially higher than those typically found in patients' blood. One possible interpretation of our findings is that in patients supplementation with vitamin D may require achieving substantial systemic levels, if the aim is to control excessive airway inflammation.

Interestingly, in 'non-responder' MDM, we observed some potentiation of stimulated IL-8 response, most obvious when the relative IL-8 change was considered. There have been reports of a similar potentiation of IL-8 production by 1,25(OH)₂D₃ in a monocytic leukaemia cell line THP-1, differentiated to macrophage-like cells (Ikeuchi *et al.*, 2013;

Ryynanen and Carlberg, 2013). It was suggested that this potentiation may occur as a 'by-product' of vitamin D receptor binding close to the IL-8 gene on chromosome 4, with subsequent chromatin opening and up-regulated transcription (Ryynanen and Carlberg, 2013). Therefore, not all intracellular effects of vitamin D may be anti-inflammatory. It is possible that, in any one individual, the IL-8 down-regulating efficacy of vitamin D is determined by a net effect of macrophage chromatin organization, interaction with transcription factors via the vitamin D receptor, down-regulation of innate immune receptors (Sadeghi *et al.*, 2006; Khoo *et al.*, 2011), and other intracellular factors modulated by vitamin D. Furthermore, not all beneficial effects of vitamin D may depend on its transcriptional activity, and recent reports appear to support the existence of such pathways (Giangreco and Nonn, 2013; Ito *et al.*, 2013; Li *et al.*, 2013).

In addition, the initial state of the cell treated with vitamin D metabolites could also define the resulting anti-inflammatory effect. Vitamin D metabolites do appear to cause mild non-specific up-regulation of IL-8 under certain circumstances, for example, by the chromatin reorganization discussed above. But their effects against excessive IL-8 production, such as in 'responder' MDM and CF MDM in our study, seemed to be predominantly anti-inflammatory. This suggests that the mechanisms and net results of treatment with vitamin D metabolites may be individualized. As intracellular signalling of vitamin D metabolites is complex, there may be more than one single factor that determines the extent of IL-8 down-regulation by these metabolites. Furthermore, net treatment results may also be determined by the levels of these metabolites.

Our experiments demonstrated that the hyperinflammatory MDM phenotype was not associated with circulating levels of 25OHD₃. It is possible that such an association could exist *in vivo*, but that it was lost during the *in vitro* maturation of monocytes to MDM. Further studies would be needed to test the hyperinflammatory phenotypes of blood monocytes before they are put into culture. If monocyte responsiveness to vitamin D metabolites is to be tested, this will require the use of 1,25(OH)₂D₃ to bypass their lack of *CYP27B1* expression.

The fact that two vitamin D metabolites, 25OHD₃ and 1,25(OH)₂D₃, most effectively down-regulated IL-8 in hyperinflammatory MDM may provide a clue to their anti-inflammatory mechanisms. Identification of intracellular protein(s) or pathway(s) that exaggerate the inflammatory response in specific individuals may reveal how vitamin D metabolites down-regulate the stimulated release of IL-8. Paradoxically, the observed hyperinflammatory MDM phenotype did not translate into refractoriness to other anti-inflammatory therapies, such as low concentrations of dexamethasone, in our studies. Importantly, those MDM which were 'non-responders' in terms of the down-regulation of IL-8 by the tested vitamin D metabolites, did down-regulate IL-8 when treated with low concentrations of dexamethasone. Thus, their 'non-responsiveness' was specific to the effects of vitamin D metabolites on IL-8 production. It would be of interest to determine if this 'non-responsiveness' is a reflection of the complex nature of intracellular effects of vitamin D metabolites discussed above.

Because the hyperinflammatory phenotype is not limited to CF MDM and was found in cells from some healthy donors, it would appear that an exaggerated inflammatory response is not feature unique to CF. It is possible that this hyperinflammatory phenotype is determined by genetic variability of the so-called inflammatory ‘modifier’ genes (Guillot *et al.*, 2014) that may be present in the general population as well. *DUSP1*, the anti-inflammatory gene up-regulated by 25OHD₃ and 1,25(OH)₂D₃, did not appear, in our experiments, to be such a gene. Hyperinflammatory MDM expressed normal levels of *DUSP1* under basal conditions and efficiently up-regulated this gene when treated with the vitamin D metabolites 25OHD₃ and 1,25(OH)₂D₃. Furthermore, this hyperinflammatory phenotype was expressed when MDM were tested with bacterial virulence factors, such as sterile filtrates of *P. aeruginosa* or *P. aeruginosa* flagellin, but not when the MDM were stimulated with the pro-inflammatory cytokine IL-1β. This could be another clue to understanding hyperinflammation in MDM.

As down-regulation of IL-8 by 25OHD₃ and 1,25(OH)₂D₃ was present only in hyperinflammatory MDM from specific individuals and only manifest with high concentrations of these metabolites, this may explain the mixed results from a recent interventional study with vitamin D supplementation in CF (Grossmann *et al.*, 2012). It is therefore important to be able to identify, prospectively, the potential ‘responders’ to the anti-inflammatory therapy with vitamin D. Furthermore, as the hyperinflammatory phenotype and down-regulation of IL-8 by vitamin D metabolites are not specifically associated with CF, it is possible that there could also be ‘non-responders’ among CF, explaining the variability of responses observed in interventional studies with vitamin D supplementation in CF. We believe that the results of our study have contributed important points of discussion in CF research.

There are some important questions that remain unanswered. First, our study did not address the long-term reproducibility of the hyperinflammatory MDM phenotype that responded the most to the anti-inflammatory effects of vitamin D. Furthermore, we did not study how macrophage inflammatory responses would be affected by their maturation in the presence of high concentrations of 25OHD₃ or 1,25(OH)₂D₃. The effects of vitamin D on macrophage IL-8 production that were studied here can be described as ‘acute’, as the overall incubation time with these vitamin D metabolites did not exceed 48 h. It is still possible that these vitamin D metabolites, 25OHD₃ and 1,25(OH)₂D₃, may also modulate inflammatory responses by affecting the macrophage phenotype during maturation. In addition, the focus of this study was on IL-8, the chemokine propagating airway inflammation. However, macrophages produce many other cytokines and chemokines, apart from IL-8. We did not test here whether other macrophage-derived inflammatory factors would be affected by vitamin D metabolites 25OHD₃ and 1,25(OH)₂D₃. Further studies should study the effects of vitamin D metabolites on these other inflammatory cytokines or chemokines. Finally, the beneficial effects of vitamin D also include stimulation of antibacterial defences, as mediated by the up-regulation of the antibacterial gene *hCAP18*. A diminished bacterial load would indirectly decrease airway inflammation.

In conclusion, we have demonstrated that high concentrations of 25OHD₃ and 1,25(OH)₂D₃, and the synthetic analogue paricalcitol, moderately down-regulate IL-8 in hyperinflammatory macrophages from specific individuals, including those from patients with CF. We further demonstrated that the down-regulation of IL-8 by these vitamin D metabolites and analogues was not associated with up-regulation of the vitamin D responsive anti-inflammatory gene *DUSP1*. Identification of the factors that cause the observed heterogeneity of the down-regulation of IL-8 by vitamin D will help individualize vitamin D supplementation in CF and extend its beneficial effects to modulation of excessive airway inflammation.

Acknowledgements

N. D. acknowledges funding from US Cystic Fibrosis Foundation, Cystic Fibrosis Canada and Kriebel Foundation. K. H. was a recipient of the Quebec Respiratory Health Training Programme (CIHR/FRSQ) and a scholarship of the Research Institute of MUHC. M. D. was the recipient of a scholarship from Cystic Fibrosis Canada. S. R. acknowledges funding from Cystic Fibrosis Canada and CIHR. L. C. L. acknowledges funding from US Cystic Fibrosis Foundation and Kriebel Foundation. The authors would like to thank R. Reiss, M. Cammisano, A. Allard-Coutu, S. Singh and C. Cr  peau for their help with carrying out this study. The authors further wish to thank Dr J. H. White for helpful comments and discussions. The kind gift of paricalcitol (Zemplar) by AbbVie was highly appreciated.

Author contributions

N. D. and L. C. L. conceived the study and developed its research design. N. D. contributed to acquisition, analysis and interpretation of data. K. H. contributed to acquisition, analysis and interpretation of data. M. D. and H. C. contributed to acquisition of data. J. B. and E. M. recruited patients with CF. J. B. and S. R. prepared sterile filtrates of *Pseudomonas aeruginosa* culture. L. C. L. recruited patients with CF, and contributed to the analysis and interpretation of data. N. D. drafted the paper. K. H. and L. C. L. critically revised the paper and the concept presented in it. All co-authors have contributed to and approved the submitted version of the paper.

Conflict of interest

The authors have no conflicts of interest to disclose. None of the authors have been or are consultants for AbbVie.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

<http://dx.doi.org/10.1111/bph.13249>

Figure S1 Up-regulation of IL-8 production by bacterial virulence factors.

Figure S2 Responsiveness of 'non-responder' and 'responder' cells to low-dose dexamethasone.